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Retention of microbiota diversity by lactose-free milk in a mouse model of elderly gut microbiota

Alexandra Ntemiri [†,‡], Celine Ribiere [†,‡], Catherine Stanton [‡,§], R. Paul Ross [†,‡], Eibhlís M. O'Connor[‡,#,⊥] Paul W. O'Toole [†,‡].

[†]School of Microbiology, Food Science Building, University College Cork, Western Road, T12 Y337 Cork, Ireland

[‡]APC Microbiome Ireland, University College Cork, Western Road, T12 Y337 Cork, Ireland

[§] Teagasc, Moorepark Food Research Centre, Fermoy, Co Cork P61 C996, Ireland.

[#]Department of Biological Sciences, School of Natural Sciences, University of Limerick, V94 T9PX, Limerick, Ireland.

[⊥]Health Research Institute, University of Limerick, V94 T9PX, Limerick, Ireland.

*Corresponding author: Tel: +353-21-490-3000; Fax: +353 21 490 3997; E-mail: pwotoole@ucc.ie

Abstract: Prebiotics may improve ageing-related dysbiosis. Milk is a source of nutrients including oligosaccharides whose prebiotic potential remains largely unexplored. We used a murine model to explore the effect of milk products on high diversity and lower diversity faecal microbiota from healthy and frail elderly subjects, respectively. Mice were treated with antibiotics and subsequently "humanised" with human faecal microbiota. The mice received lactose-free or whole milk, glycomacropeptide, or soy protein (control) supplemented diets for one month. The faecal microbiota was analysed by 16S rRNA gene amplicon sequencing. Lactose-free milk diet was as efficient as the control diet in retaining faecal microbiota diversity in mice. Both milk diets had a significant effect on the relative abundance of health-relevant taxa (e.g. Ruminococcaceae, Lachnospiraceae). The glycomacropeptide prebiotic activity previously observed *in vitro* was not replicated *in vivo*. However, these data indicate the novel prebiotic potential of bovine milk for human nutrition.

Key words: ageing, faecal microbiota, "humanised" mice, milk, lactose free milk, glycomacropeptide, soy protein, prebiotic potential

Introduction

During the last decade in particular, the bacterial component of the human gut microbiota has been intensively studied providing a more comprehensive understanding of the compositional and functional profile of the gut microbiota in health and disease [1]. Habitual diet, and to a lesser extent host genetics, are two factors that shape the gut microbiota [2-4]. The term dysbiosis refers to shifts in the composition, function and phylogenetic diversity of the gut microbiota of non-healthy individuals in comparison to healthy controls [1]. Metabolic disease, functional gastrointestinal (GIT) disorders and cognitive disorders have been linked to dysbiosis [1].

The low-grade chronic inflammation that characterises older age reflects the decline of immune system fitness to respond to stressors, a phenomenon called inflammaging [5]. Altered intestinal permeability and dysbiotic gut microbiota are potential inflammaging drivers [5-7]. Gut microbiota dysbiosis can be further fuelled by an inflamed GIT environment [8]. For prevention and treatment of dysbiosis and associated conditions, therapeutic interventions based on probiotics, prebiotics or faecal microbiota transplants (FMT) are being developed [1].

Bovine milk combines many nutrients including high quality protein, calcium, potassium and magnesium, vitamins such as vitamin D, fatty acids such as conjugated linoleic acid, complex sialylated oligosaccharides and glycoproteins [9, 10]. There is accumulating data pointing towards certain health benefits of milk and dairy consumption [9, 11,-12]. Importantly, milk consumption may contribute to muscle and bone density maintenance in older consumers [13, 14]. Despite the potential health benefits, studies have shown that elderly people often fail to meet current recommendations for daily dairy consumption partly due to misconceptions about lactose mal-absorption and dairy fat content [15, 16].

The effect of milk ingestion on the gut microbiota has not been extensively studied. A few recent studies have focused on the potential prebiotic effect of milk oligosaccharides on the gut microbiota. Using animal models "humanised" with infant gut microbiota, Charbonneau *et al*. [17] showed that sialylated bovine milk oligosaccharides (BMO) promoted weight gain associated with *Bacteroides fragilis* and *Escherichia coli* responsiveness to BMOs under malnutrition conditions. Karav *et al*. [18] showed that BMO released from glycoproteins could mimic human milk oligosaccharide (HMO) selectivity for *Bifidobacterium* strains in the infant gut microbiota. Boudry *et al*. [19] observed that a BMO-supplemented diet enhanced gut barrier function, increased caecal and colonic microbiota diversity and *Lactobacillus* relative abundance in a murine model of diet-induced obesity.

Glycomacropeptide (GMP) is a by-product of cheese making and it is released in whey protein from the kappa-casein fraction of milk by the activity of chymosin [20]. Glycomacropeptide has five mucin-type glycans, high content of sialic acid, high content of branched chain amino acids (BCAA) and essential amino acids (EAA) and is deficient in of aromatic amino acids [20]. Glycomacropeptide consumption is associated with a number of health benefits including anti-inflammatory and anti-GIT pathogen activity, and it is currently being used as an alternative source of protein for phenylketonuria (PKU) nutritional management [21, 22].

There is inconclusive data on the prebiotic potential of GMP. Selective growth promotion of *Bifidobacterium* and *Lactobacillus* by GMP has been reported based on tests in pure cultures but the selectivity failed to be confirmed when complex faecal microbiota communities were tested *in vitro* [23-25]. Some *in vivo* studies showed that GMP administration increased the colonic abundance of *Lactobacillus* and *Bifidobacterium* [26, 27]. Sawin *et al*. [28] reported that a GMP-supplemented diet significantly decreased the caecal and colonic relative abundance of

Desulfovibrio and Proteobacteria in wild type and PKU mice. Interestingly, GMP selectivity for promoting growth of Bacteroidetes taxa has also been reported [27, 28].

In this study, conventional mice were treated with a cocktail of antibiotics to deplete the gut microbiota before receiving faecal microbiota from either a healthy or a frail elderly donor. The effect of a diet supplemented with either lactose-free milk, whole milk, GMP or soy protein (control) on the transplanted faecal microbiota of elderly humans was then investigated in these "humanised" mice.

Materials and Methods

Animals and experimental design

The Health Products Regulatory Authority (HPRA) of Ireland under the European Union Regulations authorised this project with authorisation number AE19130/P033. Nighty-six 6 week-old female and male C57BL/6 mice were purchased from Envigo RMS Ltd, Oxon, UK and caged in groups of six of the same sex. The mice were weighed during the first week and every subsequent week of the trial.

The mice were acclimatised to the animal facility for one week before the commencement of the six weeks antibiotics (Abx) treatment for the depletion of the indigenous murine gut microbiota. Ampicillin (1g/L; Sigma Aldrich, St Louis, USA), Metronidazole (1g/L; Molekula, Darlington, UK), Vancomycin (500 mg/L; Molekula, Darlington, UK), Imipenem (250 mg/L; Molekula, Darlington, UK) and Ciprofloxacin-HCl (200 mg/L; Santa Cruz Biotechnology, Dallas, USA) were diluted in tap water and filter-sterilised before being given to animals as described before [29]. Animals had *ad libidum* access to Abx-water. Limited administration of HydroGel (ClearH2O.com) during the Abx treatment was used for the prevention of weight loss. Animals that lost more than 35% of their body weight were euthanised. One-day wash-out with filtered tap-water followed the Abx treatment.

Subsequently, the animals were "humanised" for three days with faecal microbiota from either a healthy elderly donor (EM425) or a frail elderly donor (EM297). The mice were introduced to the experimental diets on the wash-out day; the mice were ca. 14 weeks-old at the beginning of the dietary intervention. The duration of the dietary intervention was one month. Faecal samples were taken on the first day of acclimatisation (baseline), throughout the Abx treatment (time points T1 and T2), one week after humanisation (T3), in the mid-part of the dietary intervention (T4) and at the end of the trial (T5) (**Figure S1**).

Six male and 6 female mice were allocated to each diet (**Table S1**). The animals were fed maintenance diet (ssniff, Spezialdiäten GmbH, German) during the acclimatisation period and during the Abx treatment. The experimental diets (ssniff, Spezialdiäten GmbH, Germany) were supplemented with one of the following powders: **i. lac-free diet:** 20% lactose-free whole milk powder (Valio Ltd., Helsinki, Finland); **ii. wmilk diet:** 20% whole milk powder (Valio Ltd., Helsinki, Finland); **iii. GMP diet:** 20% CGMP-10 (Arla Food Ingredients, Denmark); **iv. control diet:** 20% soy protein isolate (provided by ssniff, Spezialdiäten GmbH, Germany). In order to achieve the isoenergetic and isonitrogenous profile of the test diets, all diets were additionally supplemented with soy protein isolate (**Table 1)** which was the basic protein ingredient for the maintenance diet. The complete compositional data content of the experimental diets is provided in **Appendix A**. All diets were sterilized by irradiation. The lactose-free milk powder (Valio Ltd., Helsinki, Finland) was produced from whole milk powder (Valio Ltd., Helsinki, Finland) by partial removal of lactose using filtration and by enzymatic hydrolysation of the remaining lactose to glucose and galactose.

Faecal slurry preparation and mouse "humanisation"

One faecal sample from a healthy 81 yr old (EM425; community "COM" type microbiota) and one from a frail 82 yr old subject (EM297; longstay "LS" type microbiota) were collected under the approval of the local Clinical Research Ethics Committee. The samples were processed under anaerobic conditions for the preparation of 10% w/v faecal slurries in PBS with 20% glycerol. The faecal slurries were kept in aliquots at -80°C and thawed in the anaerobic cabinet before gavage to mice. The animals received faecal slurry over three days by oral gavage from a total of 300 µl.

Faecal sample collection and genomic DNA extraction

Faecal samples were collected during the trial at various time points (T0 to T5) as indicated in **Figure S1.** One to two faecal pellets were collected at each time point and were immediately frozen in liquid nitrogen before being transferred to -80°C. Total DNA was extracted from murine faecal pellets and caecum content using the QIamp Fast DNA Stool (Qiagen, Manchester, UK) kit. Murine pellets were weighed before performing the extraction using the QIamp Fast DNA Stool (Qiagen, Manchester, UK) extraction kit protocol. The samples were placed in sterile tubes containing 0.1 mm, 0.5 mm and 1.0 mm zirconia/glass beads (Thistle Scientific, UK). Eight hundred ml of InhibitEX buffer was added to the samples and then homogenised under one pulse of 1 min and a final of 30 sec using a Mini-Beadbeader (Biospec Products, USA). In the intervals of the homogenisation steps the samples were placed on ice for 1 min. The samples were then placed in a 95°C heat-block for 5 min. The subsequent steps of the DNA extraction were carried out as described in the Qiagen protocol using 15 µl of proteinase K with 200 µl of AL buffer, 200 µl of lysate and 200 µl of ethanol at the relevant steps.

Library preparation for 16S rRNA gene amplicon sequencing

The V3/V4 variable region of the 16S rRNA gene was targeted for Illumina MiSeq System sequencing (San Diego, California, USA). The region was amplified using the universal 16S ribosomal RNA gene primer pair S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') / S-D-Bact-0785-a-A-21 (5'GACTACHVGGGTATC TAATC C-3') 5' [30]. The Phusion High-Fidelity PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA) was used for the amplification. The PCR products were purified and the subsequent library preparation steps were performed according to the Illumina MiSeq system protocol. Dual-index barcodes were attached to the amplicon (Nextera XT V.2 Index Kits sets A and D, Illumina) and then purified using the Agencourt AMPure XP-PCR Purification system (Beckman Coutler, Inc., California, USA). The indexed amplicons were quantified using the Qubit dsDNA HS Assay Kit (Thermo Fischer Scientific, MA, U.S.A.). The pooled library containing the same concentration of each amplicon was sequenced (2 x 250 bp) on a MiSeq Illumina platform in the Teagasc Food Research Centre sequencing facility (Fermoy, Ireland).

Microbiota composition analysis

FLASH programme [31] was used to join the paired-end reads that were subsequently quality filtered in Qiime (v.1.9.1) using the split_libraries_fastq.py script [32]. Forward primer was removed using cutadapt [33] and reverse primer with QIIME's script truncate_reverse_primer.py. The USEARCH sequence analysis tool was used for further quality filtering and *de novo* operational taxonomic unit (OTU) clustering. The sequences were filtered by length, sorted by size and single unique sequences were removed. The remaining sequences were clustered into OTUs based on 97% identity. Subsequently, UCHIME with the GOLD reference database was used for chimera removal. The initial quality filtered sequences were mapped against the OTUs (97 % identity). OTU representative sequences were taxonomically classified (classify.seqs) from phylum down to genus level with a 80% confidence threshold by using the mothur suite of tools (v1.36.1) and

the RDP database (trainset 14) [34]. SPINGO was used in order to classify the OTUs at species level [35]. Alpha and beta diversity analysis was performed in QIIME on a rarefied OTU table at 1300 reads in order to include the majority of the samples collected throughout the study. The sequences were aligned using the PyNast tool [36] in Qiime in order to generate alpha (α) diversity indices i.e., Shannon, Simpson, PD whole tree, Chao1 and Observed Species, and beta (β) diversity indices i.e. Weighted UniFrac and Unweighted UniFrac.

Statistical analysis

The statistical software R (3.2.5; R Core Team) was used for statistical analysis and visualisation of data. The reads per sample were converted into relative abundances and ggplot2 (v. 2.2.1) was used for the visualisation of the bacterial composition of the microbiota. Principal coordinates analysis (PCoA) was performed with Weighted and Unweighted UniFrac distances with the made4 package (v. 1.50.1). Analysis of similarities (ANOSIM) was used (vegan 2.4-3) for the detection of significant differences in the observed clustering between the groups. A Kruskal-Wallis test followed by a Dunn's post hoc test (dunn.test 1.3.4) with Benjamini-Hochberg p value adjustment for multiple testing was applied in order to detect significant differences in the faecal microbiota diversity and taxa relative abundances due to diet at T4 and T5.Values were considered significant when Kruskal-Wallis $p<0.5$, Kruskal-Wallis $p_{adj}<0.1$ and Dunn's post hoc test $p_{adj}<0.1$. Significance indications: "●"$p_{adj}<0.1$; "*" $p_{adj}<0.05$; "**" $p_{adj}<0.01$; "***" $p_{adj}<0.001$; "****" $p_{adj}<0.0001$. Graph Pad Prism (v 5.03) was used to visualise alpha diversity scores and animal body weight and to perform a Kruskal-Wallis test on body weight values at the end of the trial.

Results and Discussion

Effects of treatment on animal body weight

Due to initial aversion of the animals to the Abx water and subsequent abstinence from chow during the first 2 weeks of the Abx treatment, some animal body weight loss was observed (**Figure S2**) and 8 animals were euthanised. The animals begun to recover their body weight after the 2nd week of Abx treatment (**Figure S2**). At the end of the trial (T5), consumption of the milk supplemented diets resulted in increased body weight compared to baseline but not significantly different to the other diets effect (**Figure S2**). Upon dissection all animals had normal spleen weight (data not shown). The number of animals in each dietary group at T5 for which the gut microbiota was analysed is shown in **Table S1**.

Characterisation of human donor microbiota and its engraftment in mice

General features of the compositional and phylogenetic diversity differences between the faecal microbiota of frail long-term care unit-residing elderly subjects and healthy community-living were described in the ElderMet study [37]. The ElderMet subjects EM297 (LS) and EM425 (COM) were re-enrolled and their faecal microbiota was re-analysed in order to confirm suitability for the mouse trial. Compositional differences at taxon level between COM and LS type faecal microbiota have been described before [25]. Compared to the COM type microbiota, the LS microbiota had lower Firmicutes relative abundance, and it was enriched in Proteobacteria, Synergistetes and Bacteroidetes (**Figure 1**), but as expected for a subject of this type, the alpha diversity was lower (**Table S2**). Thus, the inocula were considered suitable for proceeding with mouse humanisation.

One week after humanisation (T3), the murine faecal microbiota had diverged from baseline and did not revert back to the original murine phylogenetic profile by the completion of the trial (T5), as demonstrated by analysis of the Weighted and Unweighted UniFrac distances at time-point 3 (**T3; Figure S3)** and T5 (**Figure 2**). Importantly, the COM or LS type phylogenetic separation in β-diversity was retained at T5 as shown by PCoA of the

Unweighted UniFrac distances (**Figure 2B**) in spite of the effect of diet that drove the abundance-based clustering (**Figure 2A**). The faecal microbiota from mice fed the wmilk and the lac-free diets clustered closer together compared to the mice fed the control or GMP diets irrespective of humanisation microbiota type (**Figure 2A**). A detailed description of the differential effect of the diet supplements on the faecal microbiota follows further below. Importantly, the murine host may have played a role in re-shaping the relative abundances of taxa in the xenomicrobiota (i.e. the human microbiota, LS or COM type). It has been reported that the gut of germ-free or antibiotic-treated mice may be refractory to *Clostridium* clusters XIVa and IV taxa colonisation but more susceptible to Bacteroidetes colonisation [38]. The Bacteroidetes and especially Porphyromonadaceae enrichment across most dietary groups at T5 and the compositional profile of the Lachnospiraceae at T5 comprised of mostly two taxa groups i.e. unclassified *Clostridium* cluster XIVa and unclassified Lachnospiraceae (**Figure S5**), can be viewed as the result of selective pressure applied by the murine host to reshape the relative abundances of the shared with the human xenomicrobiota taxa to best fit murine host physiology and immunity [39, 40]. *Barnesiella intestinihominis* and *Parabacteroides goldsteinii* were the most abundant Porphyromonadaceae taxa in the microbiota of COM type humanised mouse whereas *Parabacteroides merdae* was the most abundant Porphyromonadaceae in LS type humanised mouse (**Figure S5)**.

The lactose-free milk supplemented diet sustained higher microbiota diversity

In mice colonised with either human microbiota type, the lac-free diet sustained significantly higher faecal microbiota diversity compared to the wmilk or GMP diets, performing as efficiently as the soy protein supplemented diet (control) (**Figure 3A** and **B**). For example, for COM type humanised mouse microbiota at T5, feeding with lac-free diet resulted in ca. 4.5 Shannon diversity index which was significantly higher compared to the other three diets, whereas the effect of the lac-free diet feeding on indices PD (ca. 8) and Observed Species (ca.

90) was similar to that of the control diet (**Figure 3A**). A similar trend was observed for LS type humanised mouse microbiota; upon the lac-free diet the Shannon diversity index was ca. 4.1, the PD was ca. 5.5 and the Observed Species index was 58 (**Figure 3B).** Some animal and human studies indicate the prebiotic potential of soy and its products [41]. Decreased gut microbiota diversity is a biomarker of ageing and frailty, and dietary supplements contributing to sustaining diversity may slow down ageing-related health loss [37, 42]. The efficiency of the lac-free milk diet in retaining microbiota diversity can be attributed partly to the BMOs available for microbiota fermentation. Bovine milk oligosaccharides have attracted interest as potential prebiotics due to their structural complexity and similarity to HMOs [43, 44]. The effect of the diets on the diversity of the faecal microbiota of female and male mice analysed separately was similar to that presented here for the aggregated microbiota across gender (data not shown). The alpha diversity indices Chao 1 and Simpson were also analysed and indicated similar significant results as those presented in **Figure 3** (data not shown).

Differential effect of dietary supplementation on microbiota composition

Diet had a significant effect on the composition of the murine faecal microbiota as shown in **Figure 2**. In order to profile the detailed effect of the diets on the faecal microbiota of mice at T5, PCoAs of the UniFrac distances for the faecal microbiota per humanisation microbiota type were generated, i.e. COM type humanisation (**Figure 4 A** and **B**) and LS type humanisation (**Figure 4 C a**nd D). The ANOSIM test result confirmed the significant effect of the diets on the β-diversity (**Figures 4**). Within the diet-based clustering, the mouse gender had a significant impact on further sub-clustering of the microbiota in most of the dietary groups (gender is denoted by shape in **Figures 4)**. The ANOSIM test result showed that the effect of gender was significant ($p<0.05$) for most of the dietary groups and that the variation observed was due to the gender effect ($R\approx1$) (**Table 2**). Gender did not have a significant

effect on the GMP and lac-free dietary groups for LS colonised mice (Weighted and Unweighted, respectively) (**Table 2**; **Figure 4 C a**nd D).

Prebiotic potential of the lactose-free milk

The murine faecal microbiota (either colonisation type) was enriched in Ruminococcaceae upon receiving the lac-free diet (17% and 4.6% average relative abundance, COM and LS type colonisation, respectively) (**Figure 5, S4**). The responsiveness of the Ruminococcaceae in the female mice was comparable to control diet ($p>0.1$) (**Figure 6A** and **7A**), whereas in the male mice the Ruminococcaceae responsiveness to the lac-free diet was either comparable to wmilk diet ($p>0.1$) (COM type colonisation: **Figure 6B)** or significantly higher compared to all diets ($p<0.09$) (LS type colonisation; **Figure 7B**). The Ruminococcaceae enrichment associated with the lac-free diet could indicate that such a dietary supplementation might contribute to colonic health and healthy ageing as suggested by previous studies for these taxa [45, 46]. In the LS humanised mice the Ruminococcaceae reached high relative abundance after the lac-free diet but not as high as in COM type humanised mice, potentially due to low starting abundance of this family in the LS microbiota (**Figures 5** and **7**). In COM type colonised female mice and LS type colonised mice *Flavonifractor plautii* was a dominant Ruminococcaceae taxon whereas *Anaerotruncus colihominis* and *Oscillibacter* taxa were differentially abundant in female and male COM type colonised mice (**Figures 8** and **S5**). Others have reported probiotic-associated *Anaerotruncus* enrichment of dysbiotic murine microbiota after Abx treatment [47] whereas *Oscillibacter* and *Flavonifractor* taxa have been associated with resistant starch degradation and identified in the gut microbiota of lean individuals [48, 49]. However, caution is needed before extrapolating these findings to human dietary interventions because some studies

277 report enrichment of *Oscillibacter* in cancer associated microbiota and *Anaerotruncus*
278 enrichment in the microbiota of frail elderly [50, 51].

279 **Effect of the whole milk supplemented diet on the gut microbiota**

280 The whole milk supplemented diet was less efficient than the lac-free diet in sustaining
281 microbiota diversity based on the alpha diversity indices at T5 (**Figure 3**). For COM type
282 humanised mouse microbiota, the wmilk diet resulted in ca. 4.1, 6.7 and 64 Shannon, PD and
283 Observed Species indices, respectively, that were significantly lower compared to the
284 respective indices upon the lac-free diet supplementation (**Figure 3A**). Similar results were
285 observed for LS type colonised mouse microbiota (**Figure 3B**). However, the comparative
286 increase in the relative abundance of certain taxa after the wmilk diet warrants further
287 investigation. The Ruminococcaceae family were responsive to the wmilk diet only in the
288 male mice (**Figures 5**, **6** and **7**). *Ruminococcus bromii* (6% average relative abundance;
289 detected only in the COM type colonised male mice) and other unclassified taxa were the
290 dominant Ruminococcaceae representative taxa (**Figures 8C** and **S5C**). *Ruminococcus*
291 *bromii* is a dominant gut microbiota taxon highly responsive to resistant starch, essential in
292 nutrient degradation and as such it can be a prebiotic target leading to substrate release in the
293 colon and increase in cross-feeding mediated by short chain fatty acids (SCFA) production [52,
294 53].

295 The significant effect of the wmilk diet on the relative abundance of the Erysipelotrichaceae
296 (Firmicutes) is evident in **Figure S4**. In animals with COM type colonisation, the
297 Erysipelotrichaceae were the most abundant Firmicutes family in the male mouse microbiota
298 (23% average relative abundance) with *Allobaculum* as the most abundant representative
299 taxon (22% average relative abundance) (not detected in the female mice) (**Figure 8C**),
300 whereas *Turicibacter* was dominant in female mice (1.6% average relative abundance) (not

detected in male mice) (**Figure 8B**). In animals with LS type colonisation, low in abundance Erysipelotrichaceae taxa were significantly enriched in the male mouse microbiota upon wmilk diet feeding (0.6% average relative abundance) (**Figure 7B**). *Allobaculum stercorianis* was the dominant Erysipelotrichaceae taxon in the female mouse microbiota (32% average relative abundance) similarly to what was observed in the microbiota of COM type humanised male mice that were fed the wmilk diet (**Figures 8B, S5B**). The Erysipelotrichaceae enrichment of the faecal microbiota of the wmilk diet fed mice and the responsiveness of *Allobaculum* taxa to the whole milk supplementation may also be a positive effect on the microbiota. In murine trials, high-fat diet-associated dysbiosis and impaired gut barrier function were restored after BMO supplementation correlating to *Allobaculum* (and Ruminococcaceae) enrichment of the microbiota [19; 54]. Inulin supplementation had similar effect on *Allobaculum* taxa associated with improved vascular function [55]. However, experimental extrapolation to human diet is needed due to *Allobaculum* association with thrombosis risk through trimethylamine-N-oxide (TMAO) metabolism and to other erysipelotrichial associations with the obesity phenotype in murine models [54, 55].

Interestingly, the wmilk diet, similarly to the lac-free diet, sustained the highest Lachnospiraceae relative abundance in the murine microbiota compared to the GMP and control diets. Analytically, COM type humanisation: ca. 12% and 15%, upon wmilk and lac-free diet, respectively; LS type humanisation: ca. 15.5% and 29% upon wmilk and lac-free diet, respectively (**Figures 5** and **S4**). Taxa belonging to unclassified Lachnospiraceae and unclassified *Clostridium* cluster XIVa were the representative Lachnospiraceae taxa (**Figure 8**).

Here the Acidaminococcaceae taxon *Phascolarctobacterium faecium*, an asaccharolytic bacterium, was responsive to the wmilk diet (5.6% average relative abundance in COM type

colonised mouse) and the GMP diet (5% and 6% average relative abundance in COM and LS type colonised mouse, respectively), and to a lesser extent to the control diet (1.8% and 1.1% average relative abundance in COM and LS type colonised mouse, respectively) (**Figures 8 and S5**). We have previously reported Acidaminococcaceae responsiveness to GMP supplementation in an *in vitro* colon system [25]. The Acidaminococcaceae family relative abundance was significantly increased upon wmilk diet mainly when compared to the control and lac-free diet in COM type humanised mice (**Figures 6**). In LS colonised mice, the relative abundance of the Acidaminococcaceae increased significantly upon GMP diet in the female mice (**Figure 7A**); the Acidaminococcaceae family was also abundant in the male mouse microbiota (**Figure 5B** and **C; Figure S4B**). In previous mouse model studies, the taxon *Phascolarctobacterium* was linked to soy diet-associated metabolic markers improvement [58], whereas Lecomte *et al*. reported that the taxon increased after high–fat diet [59]. Tran *et al*. [60] reported the positive association of the taxon to high fibre diet in both murine and human intervention studies.

Desulfovibrionaceae responsiveness to the dairy diets

All three milk-associated diets presented in this study had some effect on the relative abundance of the Desulfovibrionaceae family of the Proteobacteria. In animals that received COM type colonisation, the GMP diet had the strongest effect on the Desulfovibrionaceae taxon *Bilophila wadsworthia* that reached 11.5% average relative abundance upon the GMP diet (**Figure S5**). The control and wmilk diet had the weakest effect on the Desulfovibrionaceae/*Bilophila* taxa responsiveness in the female (4% average relative abundance) and male mice (1.8% average relative abundance), respectively (**Figures 6** and **8**). In LS type colonised mice, the taxon *Bilophila* was not detected and *Desulfovibrio* was the Desulfovibrionaceae representative, responsive to the lac-free diet, reaching an average 8% relative abundance (**Figures 5, 8**). Contrary to what we report in the current study, Sawin

et al. [28] reported that over a month of 20% GMP supplemented diet resulted in reduced Desulfovibrionaceae/*Desulfovibrio* relative abundance and amelioration of inflammatory markers in wild type (WT) and phenylketonuria (PKU) C57Bl/6 mice and compared to casein and amino acids supplemented diets, respectively.

Desulfovibrio spp. is a taxon negatively correlated to health in elderly people [50]. Previous studies have reported that intake of milk-derived high-fat diet resulted in *Bilophila wadsworthia* associated dysbiosis and inflammation in mouse models [61, 62]. Recently, Natividad *et al* [63] using a mouse model observed that milk fat promoted *B*. *wadsworthia*, inflammation and bile acid metabolism deregulation. Importantly, colonic health was promoted by reduced *Bilophila* relative abundances in human gut microbiota [64]. Importantly, although some mouse model studies show that milk fat may be associated with negative health outcomes associated with increased *Bilophila* abundance, other studies support non-deleterious effects of dairy fat on health [73, 74, 75]. The balance of the potentially positive and negative effects of milk diets on the faecal microbiota needs to be measured and considered in human studies.

Bacteroidetes responsiveness to the dairy diets

Phylum Bacteroidetes encompasses many fibrolytic and saccharolytic taxa [68]. The Bacteroidetes were more responsive to the dairy diets then to control. In COM type colonised mice, Bacteroidetes had ≥55% average relative abundance upon the dairy diets and 40% upon control diet, whereas for LS type colonised mice, the Bacteroidetes had ≥42% average relative abundance upon the dairy diets and 32% upon control diet (**Figure 5**). In the female mice with COM type humanisation, the families Porphyromonadaceae, Rikenellaceae and other unclassified Bacteroidales were highly responsive to the GMP diet (**Figure 6A**), whereas for either gender the Bacteroidaceae (mainly *Bacteroides thetaiotaomicron* and *Bacteroides uniformis*) were significantly increased upon the wmilk diet (**Figure 6**, **S5B**).

The Porphyromonadaceae responsiveness upon the GMP diet was high also in the male mice with COM type humanisation, whereas the Bacteroidaceae were least responsive to the GMP diet compared to the other diets (**Figure 6B**). In the LS type colonised mice, the families Bacteroidaceae, Porphyromonadaceae and Rikenellaceae were responsive to the dairy diets, whereas the Rikenellaceae were also highly responsive to the control diet (the latter response was also observed in the male animals with COM type humanisation) (**Figures S4B, 6B**). Increase in *Bacteroides* population due to GMP-supplementation has been reported in mouse model studies [27, 28]. Milk and subsequently GMP contain highly sialylated carbohydrate components [10]. Interestingly, different Bacteroidaceae taxa have been associated with sialidase activity with or without sialic acid utilisation ability [69, 70]. In the current study, compared to the other diets, feeding with the GMP diet resulted in Enterobacteriaceae enrichment in the faecal microbiota of COM type colonised mice (4% average relative abundance) (**Figures 5** and **S4A**); *Escherichia*/*Shigella* taxa were the dominant Enterobacteriaceae representatives (**Figure 8**). The sialic acid content of the GMP may have contributed to the reported comparative increase in Enterobacteriaceae relative abundance [69]. Charbonneaeu *et al*. [71] reported growth improvement in a gnotobiotic mouse and piglet models through cross-feeding between *Bacteroides* taxa that could degrade sialic acid-containing carbohydrates from sialylated BMO and secondary utilisers of sialic acid-containing compounds like *E.coli.* Importantly, a similar response upon GMP diet was not observed in LS humanised mice; the Enterobacteriaceae were more responsive to the lac-free diet (1.7% average relative abundance) (**Figures 5** and **8**). These findings in combination to existing literature may highlight the need for caution when designing the dose for human trials using milk because the sialylated carbohydrate component may induce the growth of pathobionts of the *E.coli*/*Shigella* group [71].

Enrichment of the microbiota in Verrucomicrobiaceae in some dietary groups

Enrichment of the Verrucomicrobiaceae family (*Akkermansia*) (≥38% average relative abundance) was observed at T5 upon the control diet in the faecal microbiota of COM type humanised mouse and in LS type humanised mouse microbiota upon the GMP and control diets (**Figures 5** and **8**). The values of the Shannon alpha diversity index (that measure evenness [72]) at T5 may have been biased due to the high relative abundance of *Akkermansia* (i.e. decreased alpha diversity values in the dietary groups where the *Akkermansia* bloom was observed) and for this reason the alpha diversity was analysed at an earlier time-point, i.e. in the middle of the dietary intervention (T4) (**Figure S6**) in order to confirm the effect of the lac-free diet on sustaining the microbiota diversity. The Verrucomicrobiaceae bloom was detected at T4 only for mice humanised with COM type microbiota but not in LS type humanised mice. For either colonisation type at T4, both milk supplemented diets (i.e. the lac-free and the wmilk diets) sustained significantly higher alpha diversity indices (Shannon, PD and Observed Species) compared to both GMP and control diet (**Figure S7**). The *Akkermansia* bloom could be attributed to the interruption of the Abx treatment due to HydroGel treatment in some dietary groups, to coprophagy and to cross-contamination between cages. Interestingly, Dubourg *et al*. [73] reported increased *A*. *muciniphila* colonisation in the gut microbiota of patients after broad-spectrum Abx treatment.

Translatability to human nutrition

Milk is a widely accessible dietary product, it combines an array of valuable nutrients and many recent human cohort studies have demonstrated the health benefits of dairy consumption including milk [74-76]. Importantly, BMOs may be an efficient prebiotic substrate similar to HMOs [43, 44]. We have investigated the gut microbiota modulatory potential of (lactose-free and whole) milk in a murine model. A lactose-free milk supplemented diet was found a better prebiotic candidate compared to whole milk supplemented diet as it retained higher microbiota diversity. However, the whole milk diet affected the abundance of

significant health-relevant microbiota taxa. Importantly, in LS type colonised mice, the wmilk diet sustained a higher Firmicutes relative abundance compared to the Bacteroidetes relative abundance (**Figure 5**) which may indicate the potential of milk to improve the Firmicutes dominance over the Bacteroidetes in the microbiota of frail elderly [50]. When translating the findings of the current study to humans, the fact that mice may not fully tolerate lactose [77], and that the lactose fermentation in wmilk diet fed mice may have prevented BMOs utilisation by the gut microbiota, must be taken into consideration. Importantly, we did not observe any adverse outcomes in mice consuming the wmilk diet such as diarrhoea or abstinence from eating; furthermore the stool consistency of mice fed the wmilk diet (by macroscopic observation before DNA extraction) was similar to that of mice receiving the other diets. Whether or not the two milk types, i.e., whole milk and lactose-free milk, have a differential effect on the gut microbiota of lactase persistent subjects remains to be clarified in a clinical trial. Interestingly, dairy companies employ different preparation techniques for the manufacture of lactose-free milk. Whole milk can either be filtered in order for lactose to be retained and the remaining lactose is hydrolysed enzymatically (e.g. Valio Ltd. product used here) or whole milk is enzymatically treated without prior filtering. The two methods may result in lactose-free milk with differing residual content of simple sugars that, upon consumption, may result in differential effect on the gut microbiota. Future *in vivo* studies can clarify the potential differential effect on the gut microbiota of the two preparations. Importantly, in this study, similarly to previous observations, we observed gender dependent differential responses to diet partly attributed to hormonal effects [78, 79]. Addressing gender-dependant responsiveness of the faecal microbiota to diet in murine models could increase translatability of the findings to human nutrition.

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Supporting Information Description: Table S1 Treatment groups indicating the diet, gender and the colonisation with human faecal microbiota type, Table S2 Alpha diversity indices of the faecal microbiotas of the human donors and the murine baseline faecal microbiota, Figure S1 Timeline of the mouse trial, Figure S2 Animal body weight during the 11 weeks of the trial, Figure S3 Principal coordinates analysis (PCoA) of the UniFrac distances one week after "humanisation" (T3), Figure S4 Differentially abundant taxa at family level at the end of the trial (T5) for aggregated female and male mice faecal microbiota, Figure S5 Faecal microbiota composition at species level at the end of the trial (T5), Figure S6 Composition of the murine faecal microbiota at phylum level at the mid part of the dietary intervention (T4), Figure S7 Alpha diversity indices at the mid part of the dietary intervention (T4). **Accession numbers:** Sequence Read Archive (SRA) (NCBI) under accession no. PRJNA504771.

Conflict of interest: The authors declare no conflict of interest.

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FIGURE CAPTIONS

Figure 1 Composition of the human and murine baseline faecal microbiota at family level. Bacterial families that were present at >1% relative abundance are presented. Murine: aggregated faecal microbiota across all mice at baseline; COM: community type faecal microbiota; LS: longstay type faecal microbiota.

Figure 2 Principal coordinates analysis (PCoA) of the UniFrac distances at the end of the trial (T5). A. Weighted UniFrac distances; B. Unweighted UniFrac distances. Blue: murine baseline microbiota; green colour coding: community (COM) type faecal microbiota; red colour coding: longstay (LS) type faecal microbiota. The inocula of human faecal microbiota (donors) are denoted with "▲" and the relevant red and green colour..

Figure 3 Alpha diversity indices at the end of the trial (T5). A: community (COM) type colonisation; B: longstay (LS) type colonisation. LAC_FREE: lactose free milk supplemented diet; WMILK: whole milk supplemented diet; GMP: glycomacropeptide supplemented diet; CONTROL: soy protein diet. Asterisks next to alpha diversity indices denote the result of the Kruskal-Wallis comparison; markings above boxplots denote the Dunn's post hoc result. Significant values: "●" padj<0.1; "*" padj<0.05; "**" padj<0.01; "***" padj<0.001; "****" padj<0.0001.

Figure 4 Principal coordinates analysis (PCoA) of the faecal microbiota of mice at the end of the trial (T5). Community type (COM) humanisation: A: Weighted UniFrac distances; B: Unweighted UniFrac distances. Longstay type (LS) humanisation: C: Weighted UniFrac distances; D: Unweighted UniFrac distances.LAC_FREE: lactose free milk supplemented diet; WMILK: whole milk supplemented diet; GMP: glycomacropeptide supplemented diet; CONTROL: soy protein diet."●": female mice microbiota; "▲": male mice microbiota. The ANOSIM results of the diet effect are noted.

Figure 5 Faecal microbiota composition at family level at the end of the trial (T5). COM: community type colonisation; LS: longstay type colonisation. A. microbiota composition of female and male mouse aggregated; B. female mouse microbiota composition; C. male mouse microbiota composition. LAC_FREE: lactose free milk supplemented diet; WMILK: whole milk supplemented diet; GMP: glycomacropeptide supplemented diet; CONTROL: soy protein diet. Bacterial families that were present at >1% relative abundance are presented.

Figure 6 Differentially abundant taxa at family level at the end of the trial (T5) for community (COM) type humanisation. A: female mouse microbiota; B: male mouse microbiota. Asterisks next to taxa denote the result of Kruskal-Wallis comparison; markings above boxplots denote the Dunn's post hoc result. Significant values: "●" padj<0.1; "*" padj<0.05; "**"padj<0.01; "***" padj<0.001; "****" padj<0.0001.

Figure 7 Figure Differentially abundant taxa at family level at the end of the trial (T5) for longstay (LS) type humanisation. A: female mouse microbiota; B: male mouse microbiota. Asterisks next to taxa denote the result of Kruskal-Wallis comparison; markings above boxplots denote the Dunn's post hoc result. Significant values: "●" padj<0.1; "*" padj<0.05; "**" padj<0.01; "***" padj<0.001; "****" padj< 0.0001.

Figure 8 Faecal microbiota composition at genus level at the end of the trial (T5). COM: community type colonisation; LS: longstay type colonisation. A. microbiota composition of female and male mouse aggregated; B. female mouse microbiota composition; C. male mouse microbiota composition. LAC_FREE: lactose free milk supplemented diet; WMILK: whole milk supplemented diet; GMP: glycomacropeptide supplemented diet; CONTROL: soy protein diet. Bacterial genera that were present at >1% relative abundance are presented.

Figure S 1 Timeline of the mouse trial. Time points of faecal sample collection: baseline (T0) during acclimatisation; T1 and T2 during the antibiotic treatment; T3, T4 and T5 during the dietary intervention.

Figure S 2 Animal body weight during the 11 weeks of the trial. The body weight from the first week of Abx treatment (W0) till the completion of the trial (W11) is shown. Body weight change between baseline and end of trial (T5) is shown at the bottom. A and B: COM: community type humanisation; LS: longstay type humanisation, respectively.

Figure S 3 Principal coordinates analysis (PCoA) of the UniFrac distances one week after "humanisation" (T3). A: Weighted UniFrac distances; B: Unweighted UniFrac distances. Blue: murine baseline microbiota; green colour coding: community (COM) type faecal microbiota; red colour coding: longstay (LS) type faecal microbiota. Colour shading denotes the test diets. The inocula of human faecal microbiota are denoted with "▲" and the relevant red and green colour.

Figure S 4 Differentially abundant taxa at family level at the end of the trial (T5) for aggregated female and male mouse faecal microbiota. A: community (COM) type colonisation; B: longstay (LS) type colonisation. Asterisks next to taxa denote the result of Kruskal-Wallis comparison; markings above boxplots denote the Dunn's post hoc result. Significant values: "●" padj<0.1; "*" padj<0.05; "**" padj<0.01; "***" padj<0.001; "****" padj<0.0001. COM type humanisation: Differences in the Erysipelotrichaceae relative abundance shown in Figures 6 and S4A are due to filtering for taxa presence in the compared groups for the statistical analysis. LS type humanisation: Due to filtering of the taxa tables for the statistical analysis, *Allobaculum* that was not detected in the LS type colonised male mice and fed wmilk diet (Figure 8C), was not represented in Figure 7.

Figure S 5 Faecal microbiota composition at species level at the end of the trial (T5). COM: community type colonisation; LS: longstay type colonisation. A. microbiota composition of female and male mouse aggregated; B. female mouse microbiota composition; C. male mouse microbiota composition LAC_FREE: lactose free milk supplemented diet; WMILK: whole milk supplemented diet; GMP: glycomacropeptide supplemented diet; CONTROL: soy protein diet. Bacterial species present at >1% relative abundance are shown. Colour coding: purple: Bacteroidetes; green: Firmicutes; red: Proteobacteria; blue: Verrucomicrobia.

Figure S 6 Composition of the murine faecal microbiota at family level at the mid part of the dietary intervention (T4). COM: community type microbiota; LS: longstay type microbiota. LAC_FREE: lactose free milk diet; WMILK: whole milk diet, GMP: glycomacropeptide diet; CONTROL: soy protein diet. Only phyla present at >1% relative abundance are presented.

Figure S 7 Alpha diversity indices at the mid part of the dietary intervention (T4). A: community (COM) type colonisation; B: longstay (LS) type colonisation. LAC_FREE: lactose free milk supplemented diet; WMILK: whole milk supplemented diet; GMP: glycomacropeptide supplemented diet; CONTROL: soy protein diet. Asterisks next to alpha diversity indices denote the result of the Kruskal-Wallis comparison; markings above boxplots denote the Dunn's post hoc result: Significant values: "●" padj<0.1; "*" padj<0.05; "**" padj<0.01; "***" padj<0.001; "****" padj<0.0001.

TABLES

Table 1 Composition of the customised experimental diets (ssniff Spezialdiäten GmbH).

Ingredient %	Control	20% whole milk powder	20% lactose-free milk powder	20% CGMP-10 powder
Soy protein isolate	20.0	14.5	12.75	1.8
Whole milk powder	-	20.0	-	-
Lactose-free milk powder	-	-	20.0	-
Glycomacropeptide CGMP-10	-	-	-	20.0
Maltodextrin	33.1	33.2	32.85	32.0
Sucrose	10.5	2.5	4.3	10.3
Cellulose powder	6.0	4.9	5.0	5.2
Soybean oil	7.5	2.3	2.3	7.5
Contents %				
Crude protein	18.1	18.1	18.1	18.1
Crude fat	7.6	7.6	7.6	7.6
Crude ash	4.6	5.4	5.6	5.5
Starch	31.8	31.9	31.6	30.8
Sugar (total)	12.3	12.3	12.3	12.3
Lactose	-	8.0	-	-
Energy (MJ/kg)	16.0	16.0	16.0	16.0
kcal % Protein	19	19	19	19
kcal % Fat	18	18	18	18
kcal % Carbohydrates	63	63	63	63

Table 2 ANOSIM test results for the effect of gender on the observed PCoA clustering of the faecal microbiota at the end of the trial (T5).

	COM				LS			
	Weighted		Unweighted		Weighted		Unweighted	
	ANOSIM							
	p value	R	p value	R	p value	R	p value	R
Control	0.005	0.98	0.006	1.0	0.004	0.75	0.003	1.0
GMP	0.014	0.98	0.01	0.94	0.41	0.0	0.005	1.0
Lactose-free milk	0.009	1.0	0.008	1.0	0.04	0.37	0.19	0.10
Whole milk diet	0.008	1.0	0.005	1.0	0.028	1.0	0.035	1.0

FIGURE GRAPHICS

FIGURE 1

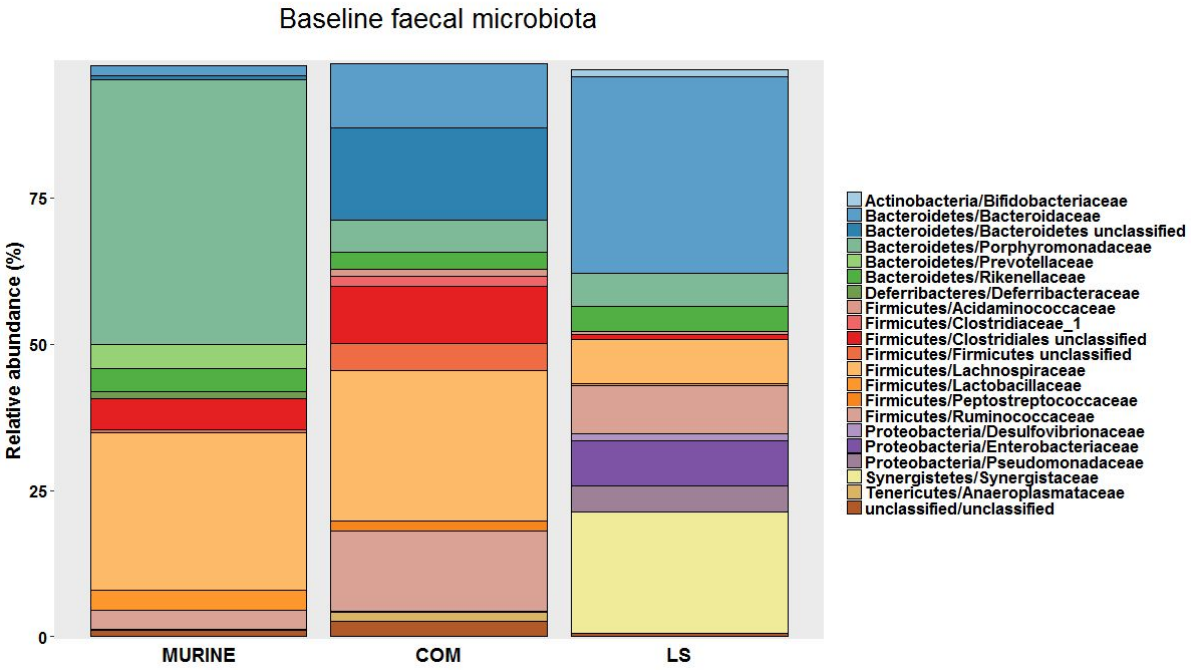


FIGURE 2

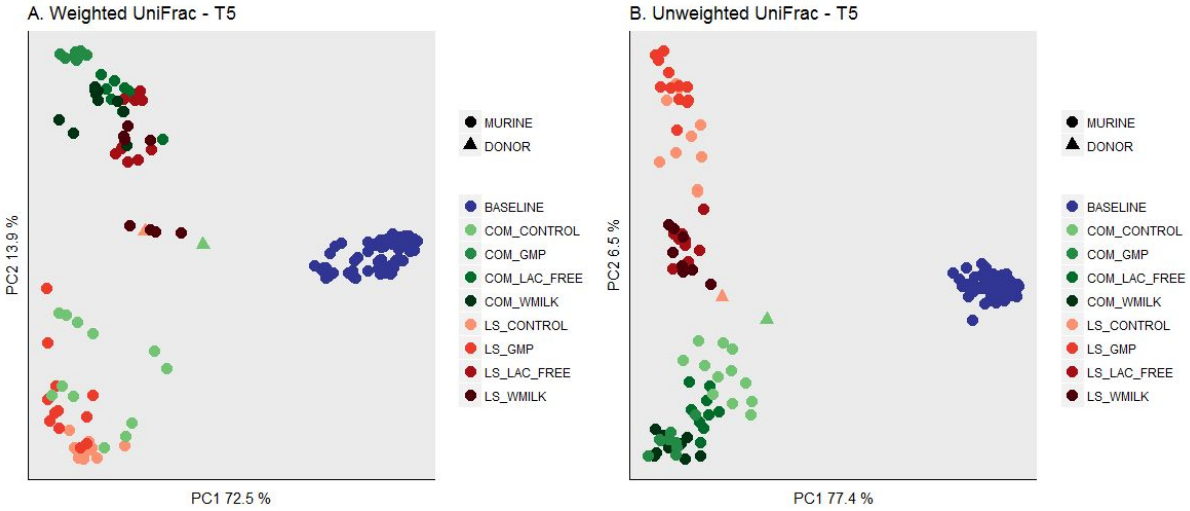

A. Weighted UniFrac - T5
PC2 13.9 %
PC1 72.5 %
B. Unweighted UniFrac - T5
PC2 6.5 %
PC1 77.4 %
MURINE
DONOR
BASELINE
COM_CONTROL
COM_GMP
COM_LAC_FREE
COM_WMILK
LS_CONTROL
LS_GMP
LS_LAC_FREE
LS_WMILK

FIGURE 3

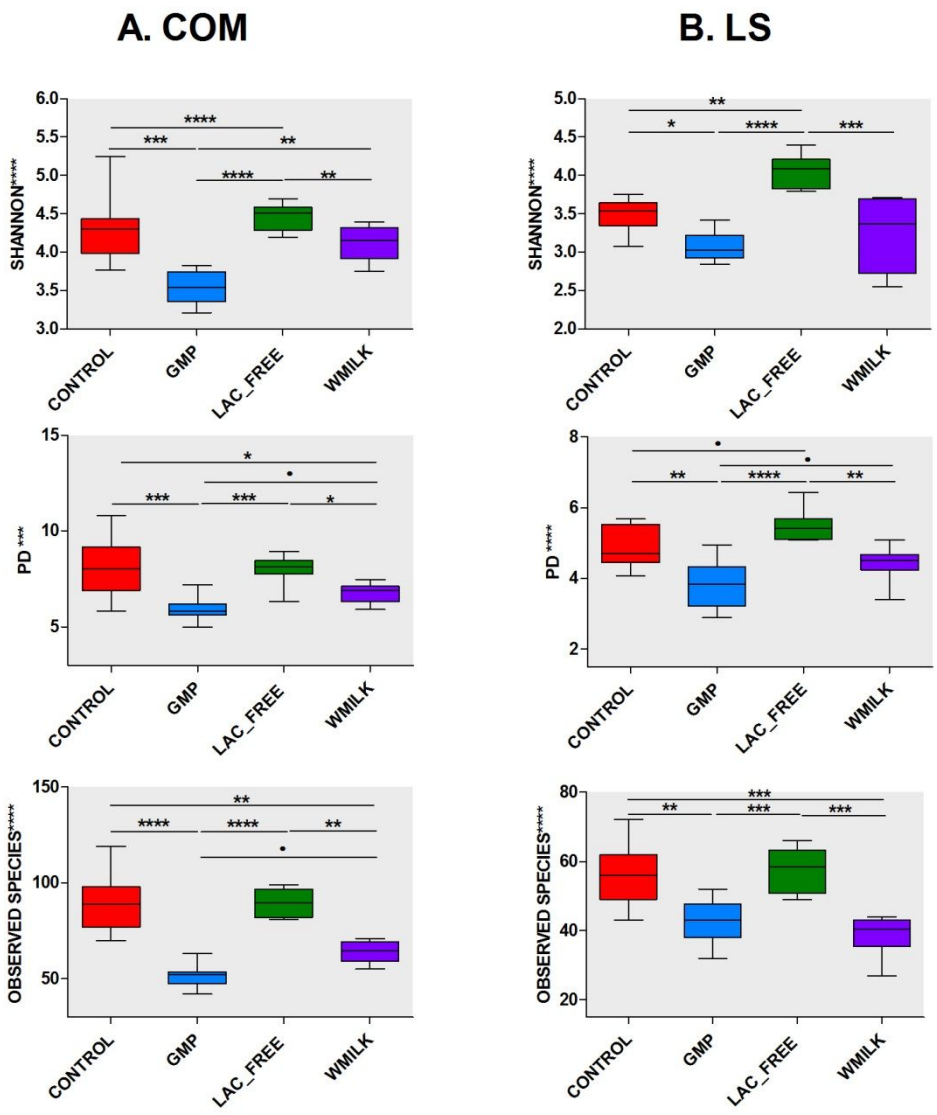

A. COM
B. LS
SHANNON****
PD ***
PD ****
OBSERVED SPECIES****
CONTROL
GMP
LAC_FREE
WMILK

FIGURE 4

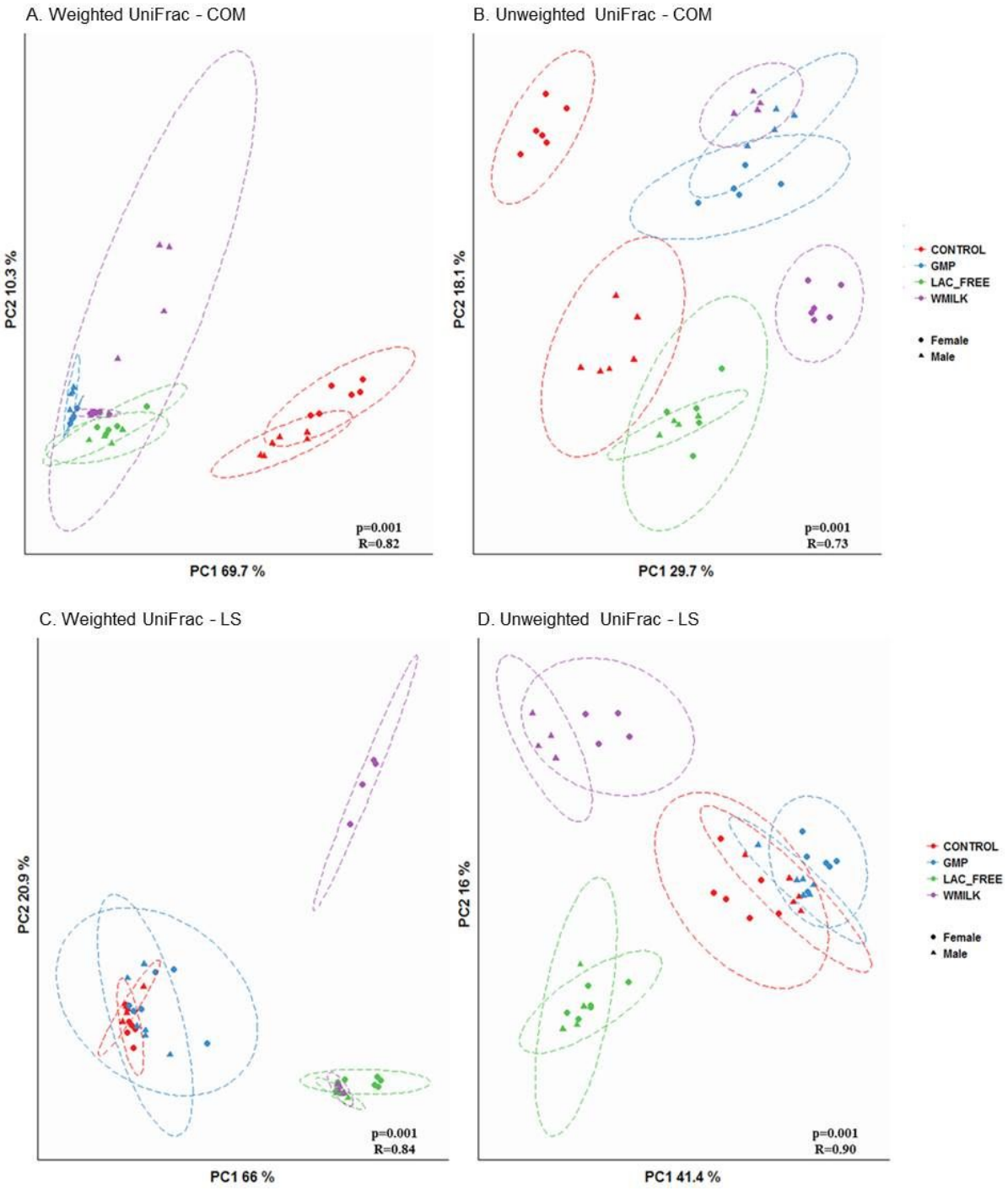

A. Weighted UniFrac - COM
PC2 10.3 %
PC1 69.7 %
p=0.001
R=0.82
B. Unweighted UniFrac - COM
PC2 18.1 %
PC1 29.7 %
p=0.001
R=0.73
CONTROL
GMP
LAC_FREE
WMILK
Female
Male
C. Weighted UniFrac - LS
PC2 20.9 %
PC1 66 %
p=0.001
R=0.84
D. Unweighted UniFrac - LS
PC2 16 %
PC1 41.4 %
p=0.001
R=0.90
CONTROL
GMP
LAC_FREE
WMILK
Female
Male

FIGURE 5

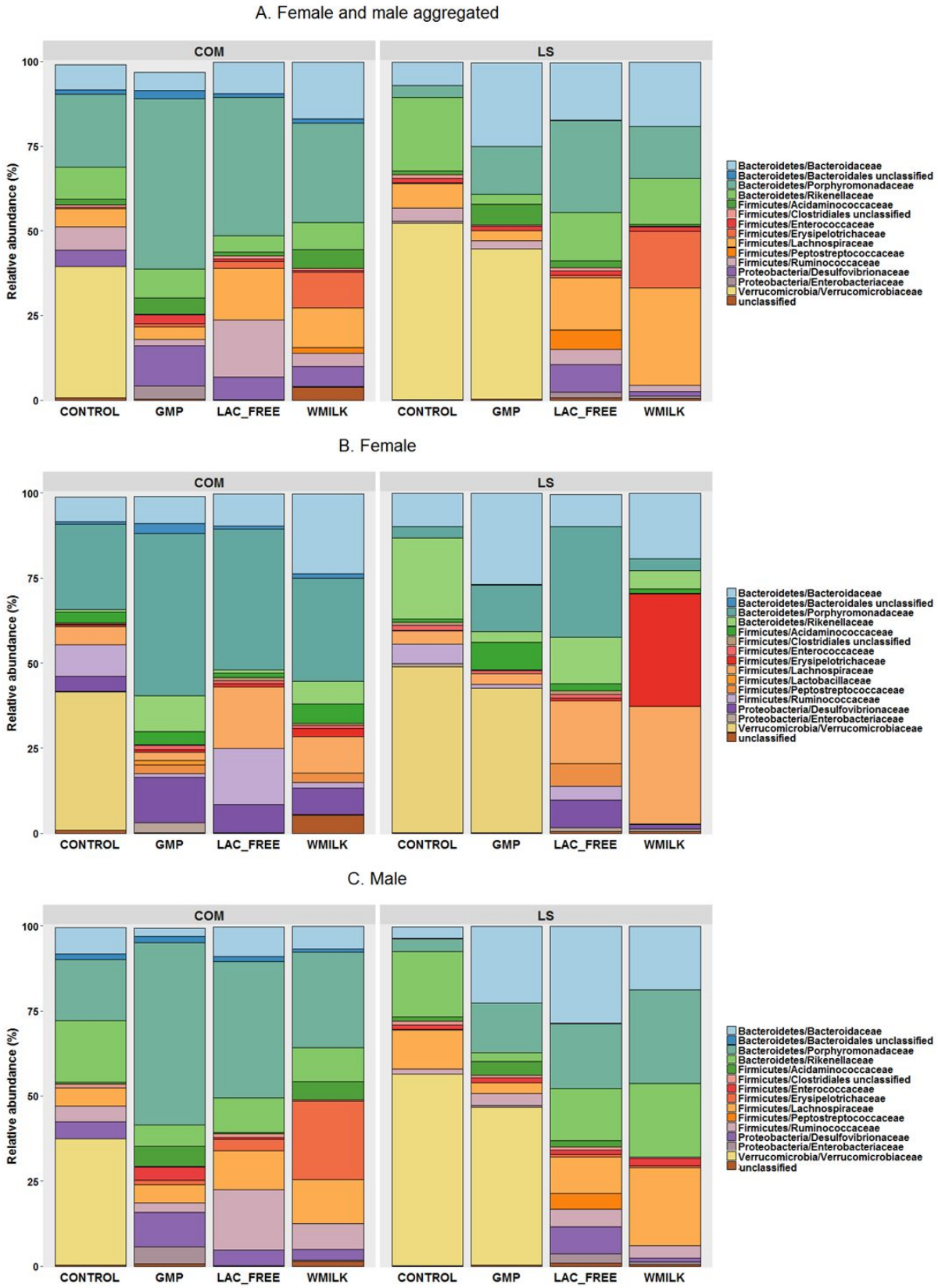

A. Female and male aggregated
COM
LS
Relative abundance (%)
100
75
50
25
0
CONTROL
GMP
LAC_FREE
WMILK
Bacteroidetes/Bacteroidaceae
Bacteroidetes/Bacteroidales unclassified
Bacteroidetes/Porphyromonadaceae
Bacteroidetes/Rikenellaceae
Firmicutes/Acidaminococcaceae
Firmicutes/Clostridiales unclassified
Firmicutes/Enterococcaceae
Firmicutes/Erysipelotrichaceae
Firmicutes/Lachnospiraceae
Firmicutes/Peptostreptococcaceae
Firmicutes/Ruminococcaceae
Proteobacteria/Desulfovibrionaceae
Proteobacteria/Enterobacteriaceae
Verrucomicrobia/Verrucomicrobiaceae
unclassified
B. Female
C. Male

FIGURE 6

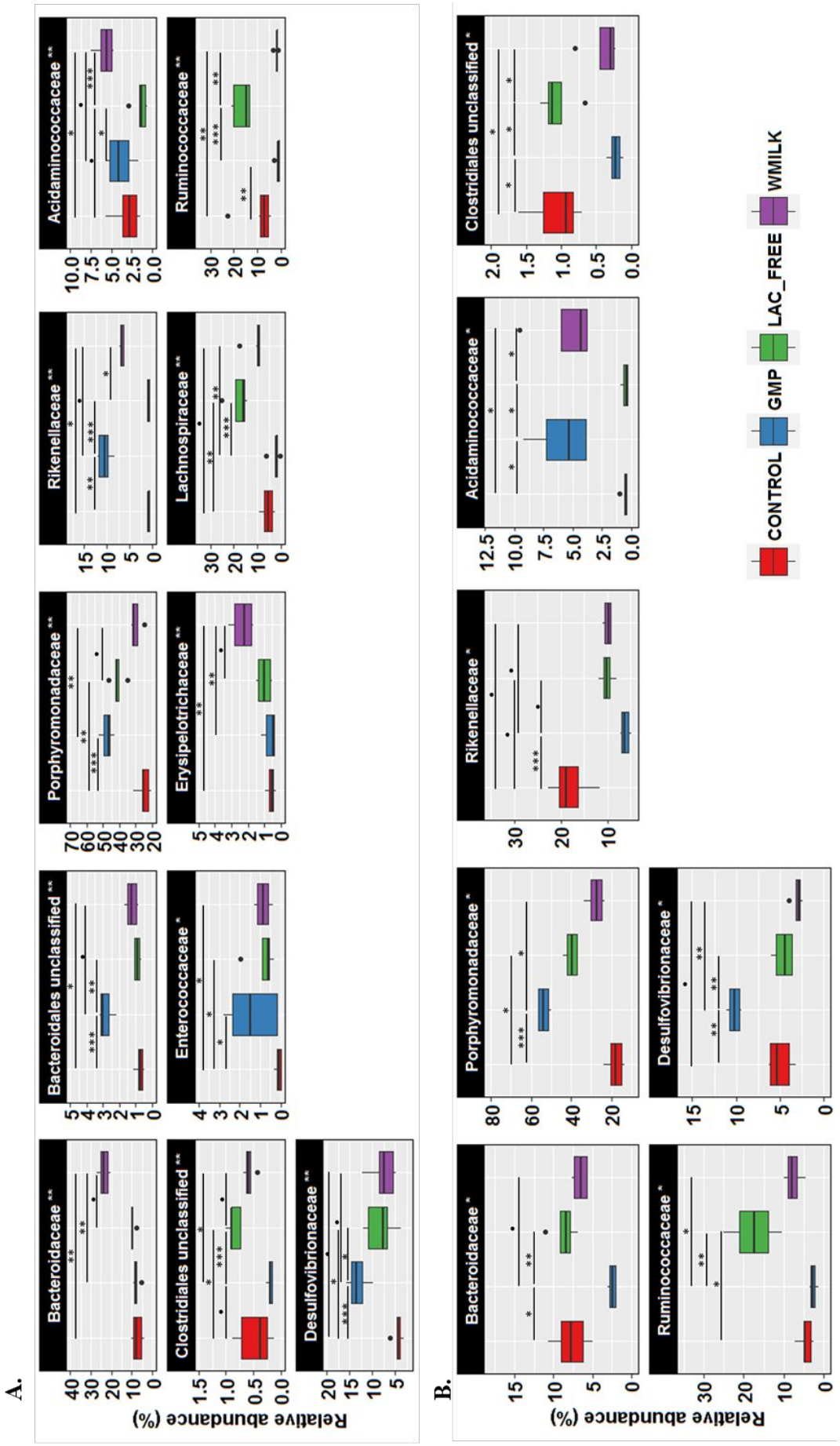

A.
B.
Relative abundance (%)
Bacteroidaceae **
Bacteroidales unclassified **
Porphyromonadaceae **
Rikenellaceae **
Acidaminococcaceae **
Clostridiales unclassified **
Enterococcaceae *
Erysipelotrichaceae **
Lachnospiraceae **
Ruminococcaceae **
Desulfovibrionaceae **
CONTROL
GMP
LAC_FREE
WMILK

FIGURE 7

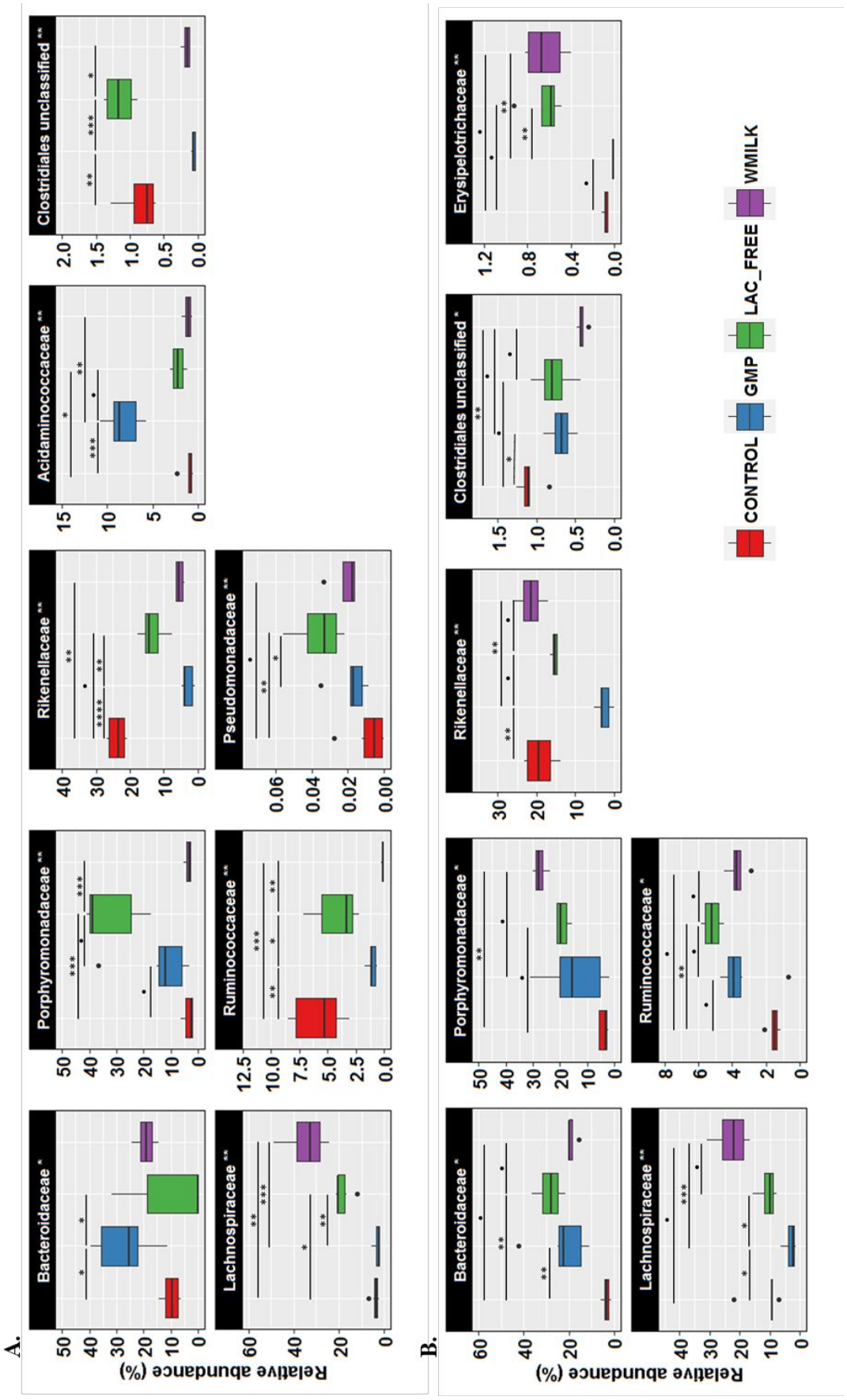
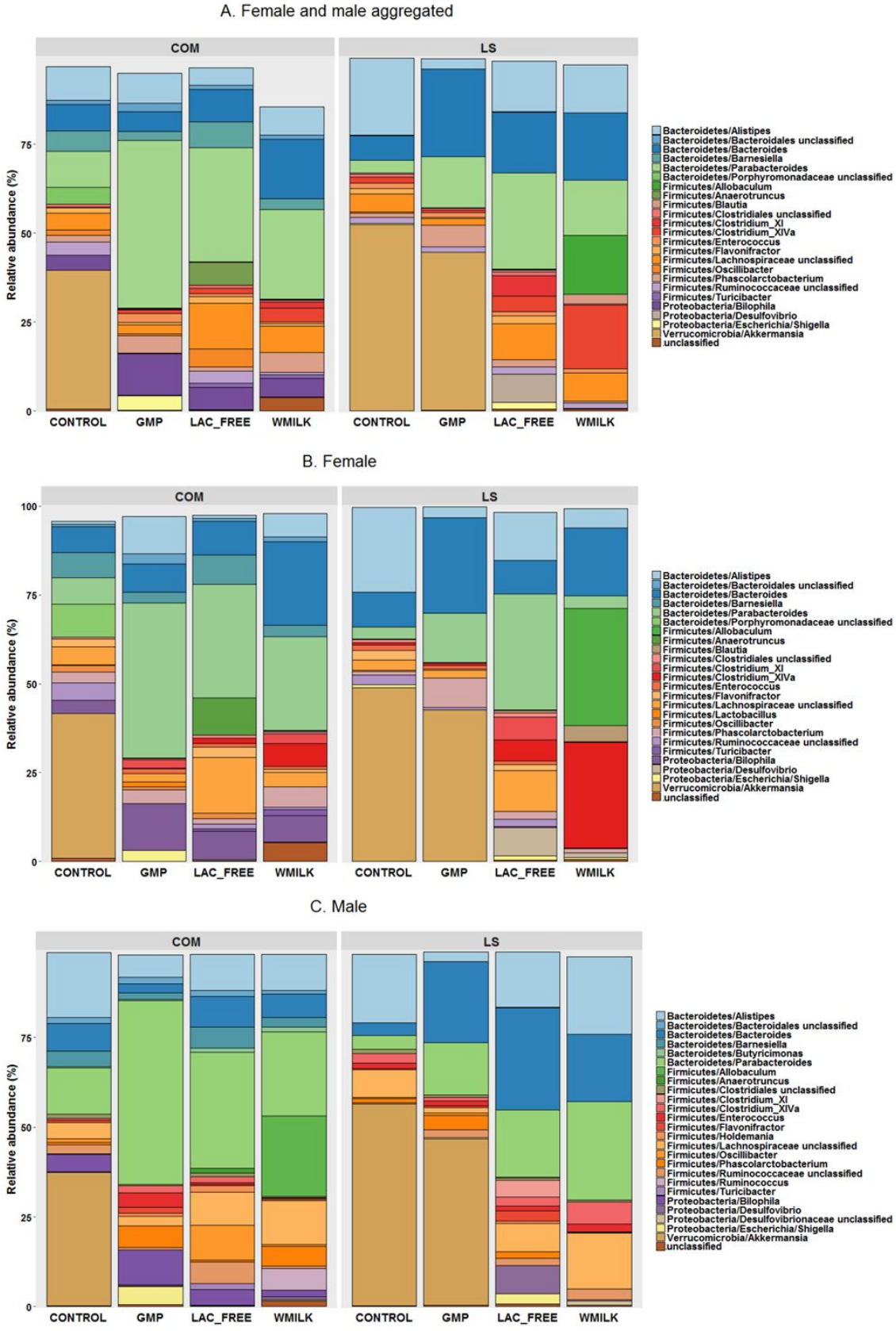

A.
B.
Relative abundance (%)
Bacteroidaceae *
Porphyromonadaceae **
Rikenellaceae **
Acidaminococcaceae **
Clostridiales unclassified **
Lachnospiraceae **
Ruminococcaceae **
Pseudomonadaceae **
Erysipelotrichaceae **
CONTROL
GMP
LAC_FREE
WMILK

FIGURE 8


A. Female and male aggregated
COM
LS
Relative abundance (%)
0
25
50
75
CONTROL
GMP
LAC_FREE
WMILK
Bacteroidetes/Alistipes
Bacteroidetes/Bacteroidales unclassified
Bacteroidetes/Bacteroides
Bacteroidetes/Barnesiella
Bacteroidetes/Parabacteroides
Bacteroidetes/Porphyromonadaceae unclassified
Firmicutes/Allobaculum
Firmicutes/Anaerotruncus
Firmicutes/Blautia
Firmicutes/Clostridiales unclassified
Firmicutes/Clostridium_XI
Firmicutes/Clostridium_XlVa
Firmicutes/Enterococcus
Firmicutes/Flavonifractor
Firmicutes/Lachnospiraceae unclassified
Firmicutes/Oscillibacter
Firmicutes/Phascolarctobacterium
Firmicutes/Ruminococcaceae unclassified
Firmicutes/Turicibacter
Proteobacteria/Bilophila
Proteobacteria/Desulfovibrio
Proteobacteria/Escherichia/Shigella
Verrucomicrobia/Akkermansia
unclassified
B. Female
COM
LS
Relative abundance (%)
0
25
50
75
100
CONTROL
GMP
LAC_FREE
WMILK
Bacteroidetes/Alistipes
Bacteroidetes/Bacteroidales unclassified
Bacteroidetes/Bacteroides
Bacteroidetes/Barnesiella
Bacteroidetes/Parabacteroides
Bacteroidetes/Porphyromonadaceae unclassified
Firmicutes/Allobaculum
Firmicutes/Anaerotruncus
Firmicutes/Blautia
Firmicutes/Clostridiales unclassified
Firmicutes/Clostridium_XI
Firmicutes/Clostridium_XlVa
Firmicutes/Enterococcus
Firmicutes/Flavonifractor
Firmicutes/Lachnospiraceae unclassified
Firmicutes/Lactobacillus
Firmicutes/Oscillibacter
Firmicutes/Phascolarctobacterium
Firmicutes/Ruminococcaceae unclassified
Firmicutes/Turicibacter
Proteobacteria/Bilophila
Proteobacteria/Desulfovibrio
Proteobacteria/Escherichia/Shigella
Verrucomicrobia/Akkermansia
unclassified
C. Male
COM
LS
Relative abundance (%)
0
25
50
75
CONTROL
GMP
LAC_FREE
WMILK
Bacteroidetes/Alistipes
Bacteroidetes/Bacteroidales unclassified
Bacteroidetes/Bacteroides
Bacteroidetes/Barnesiella
Bacteroidetes/Butyricimonas
Bacteroidetes/Parabacteroides
Firmicutes/Allobaculum
Firmicutes/Anaerotruncus
Firmicutes/Clostridiales unclassified
Firmicutes/Clostridium_XI
Firmicutes/Clostridium_XlVa
Firmicutes/Enterococcus
Firmicutes/Flavonifractor
Firmicutes/Holdemania
Firmicutes/Lachnospiraceae unclassified
Firmicutes/Oscillibacter
Firmicutes/Phascolarctobacterium
Firmicutes/Ruminococcaceae unclassified
Firmicutes/Ruminococcus
Firmicutes/Turicibacter
Proteobacteria/Bilophila
Proteobacteria/Desulfovibrio
Proteobacteria/Desulfovibrionaceae unclassified
Proteobacteria/Escherichia/Shigella
Verrucomicrobia/Akkermansia
unclassified

821

Supporting Information

Table S 1 Treatment groups indicating the diet, gender and the colonisation with human faecal microbiota type. The number of animals in each dietary group at T5 for which the gut microbiota was analysed is shown. The experimental arms of groups A, B, C and D were performed simultaneously followed by the experimental arms of groups E, F, G and H. The animals that received HydroGel treatment during Abx period, is indicated by #.

	Community type human microbiota (COM)		**Longstay type human microbiota (LS)**	
	female	**male**	**female**	**male**
Lactose free milk	A (n=5)	A (n=5)	B (n=4)	B (n=5)
Whole milk	C (n=6)	C (n=4)	D (n=4)	D (n=4)
GMP	E (n=5)#	E (n=4)#	G (n=6)	G (n=6)
Control	H (n=6)###	H (n=6)	F (n=6)##	F (n=5)###

"#","##","###" three, six and nine days, respectively of HydroGel intake

Table S 2 Alpha diversity indices of the faecal microbiotas of the human donors and the murine baseline faecal microbiota. EM297: longstay type faecal microbiota (LS); EM425: community type faecal microbiota (COM); murine: aggregated faecal microbiota across all mice at baseline.

Alpha Diversity Index	**MURINE**	**EM297 LS**	**EM425 COM**
Shannon	6.0	4.0	6.0
Simpson	1.0	1.0	1.0
PD Whole tree	15.0	9.0	17.0
Observed species	207.0	73.0	169.0
Chao 1	346.0	104.0	264.0

FIGURE S1

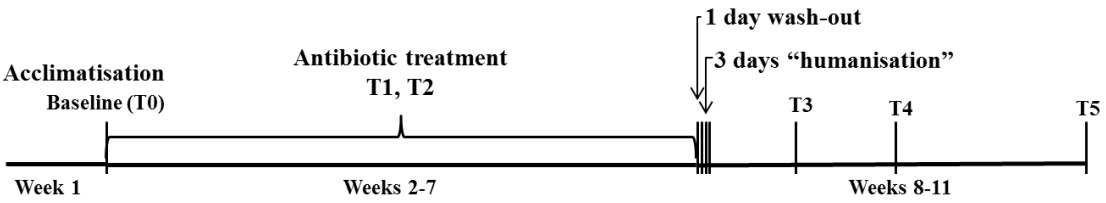

1 day wash-out
3 days "humanisation"
Acclimatisation
Baseline (T0)
Antibiotic treatment
T1, T2
T3
T4
T5
Week 1
Weeks 2-7
Weeks 8-11

FIGURE S2

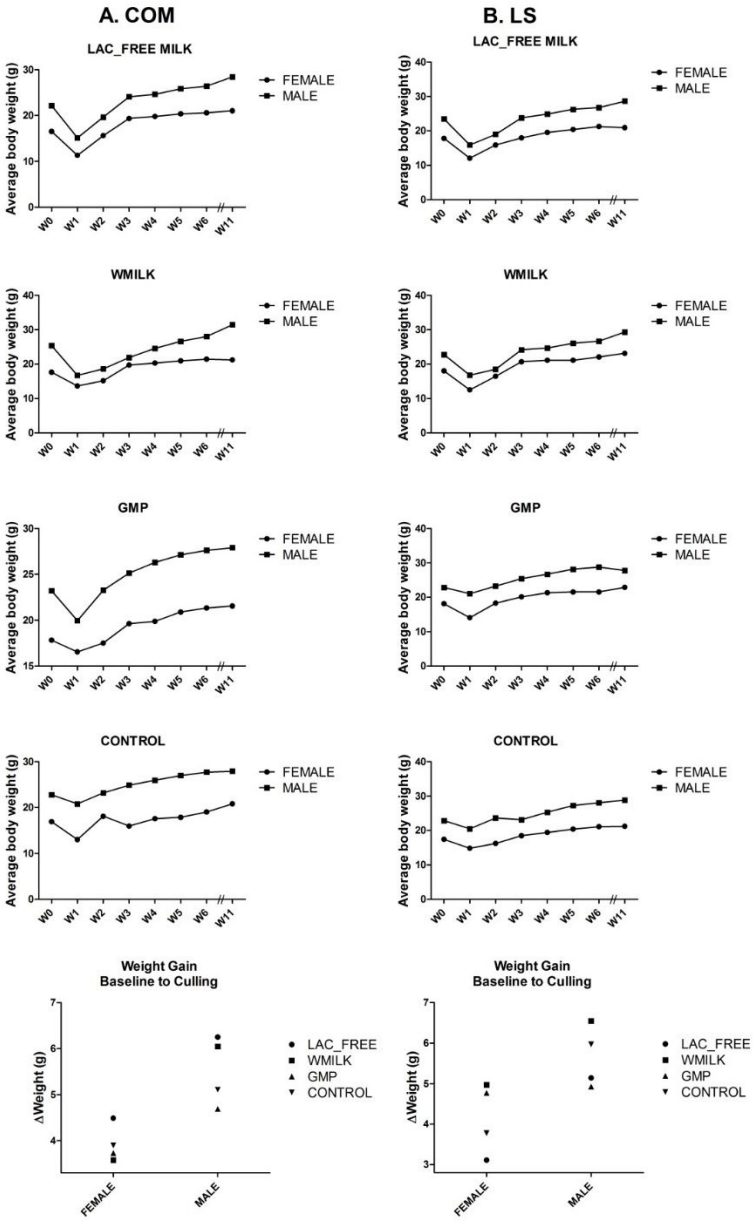

A. COM
B. LS
LAC_FREE MILK
WMILK
GMP
CONTROL
Average body weight (g)
FEMALE
MALE
W0
W1
W2
W3
W4
W5
W6
W11
Weight Gain
Baseline to Culling
ΔWeight (g)
LAC_FREE
WMILK
GMP
CONTROL

FIGURE S3

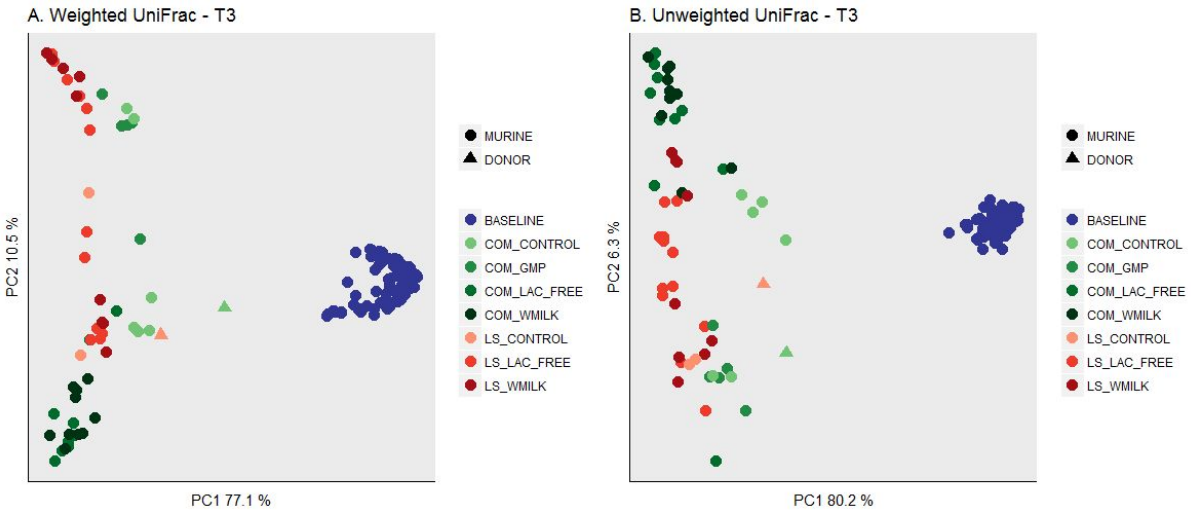

A. Weighted UniFrac - T3
PC2 10.5 %
PC1 77.1 %
B. Unweighted UniFrac - T3
PC2 6.3 %
PC1 80.2 %
MURINE
DONOR
BASELINE
COM_CONTROL
COM_GMP
COM_LAC_FREE
COM_WMILK
LS_CONTROL
LS_LAC_FREE
LS_WMILK

FIGURE S4

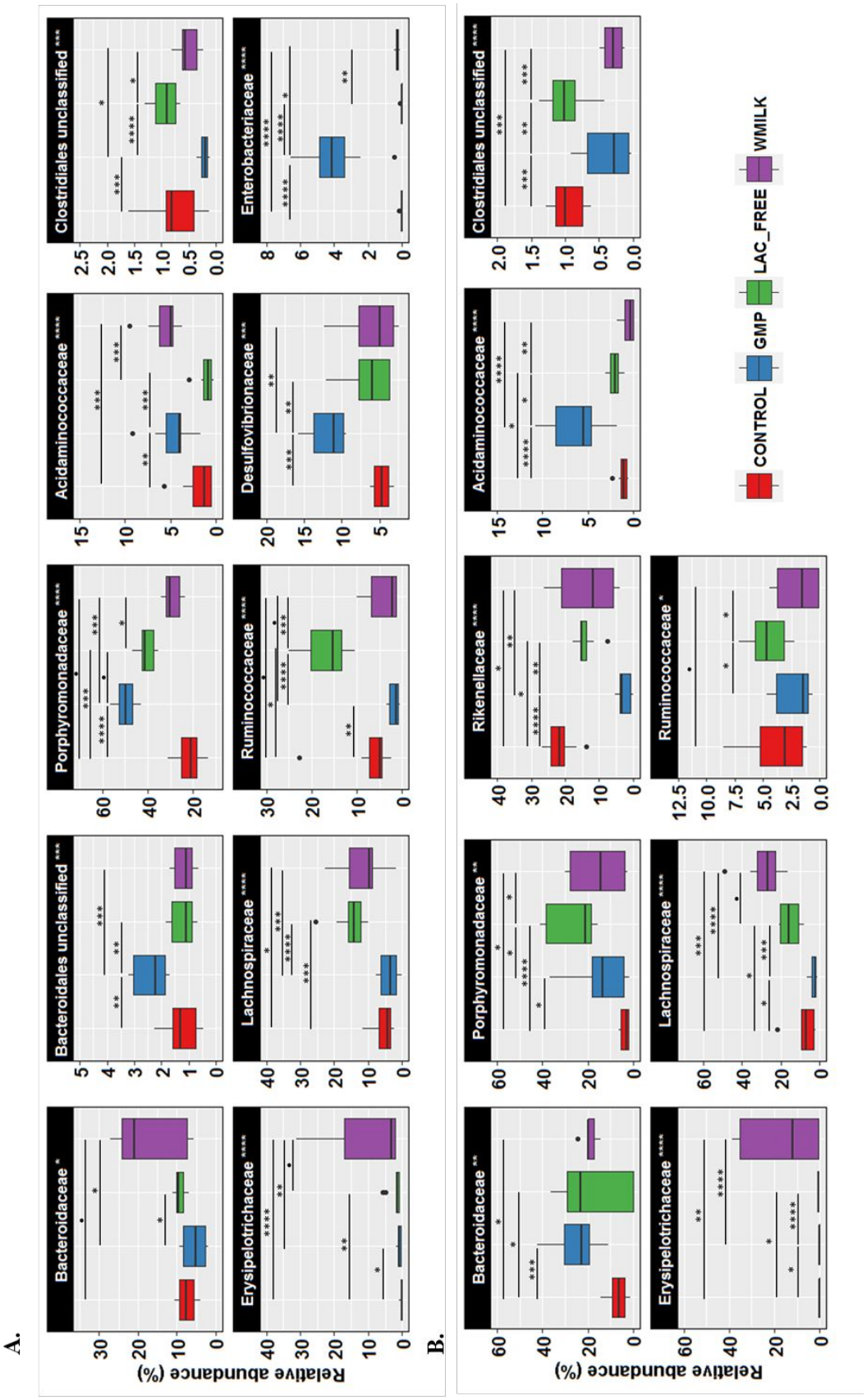
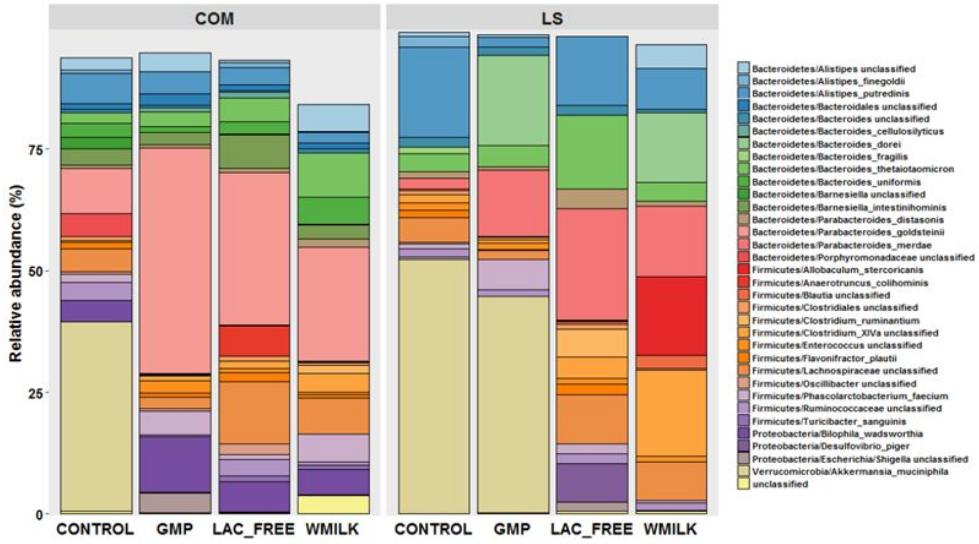
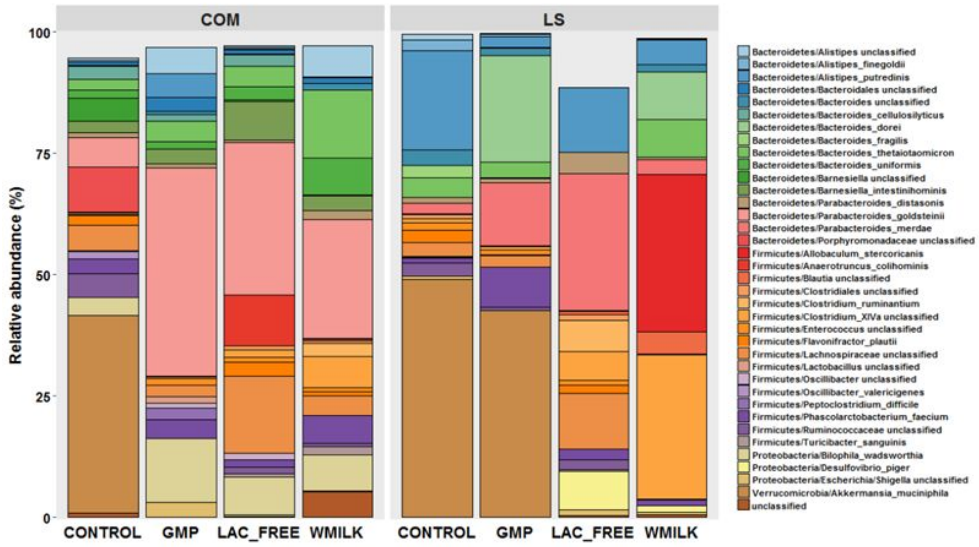

A.
B.
Relative abundance (%)
Bacteroidaceae *
Bacteroidales unclassified ***
Porphyromonadaceae ****
Acidaminococcaceae ****
Clostridiales unclassified ***
Erysipelotrichaceae ****
Lachnospiraceae ****
Ruminococcaceae ****
Desulfovibrionaceae ***
Enterobacteriaceae ****
Bacteroidaceae **
Porphyromonadaceae **
Rikenellaceae ****
Acidaminococcaceae ****
Clostridiales unclassified ****
Erysipelotrichaceae ****
Lachnospiraceae ****
Ruminococcaceae *
CONTROL
GMP
LAC_FREE
WMILK

FIGURE S5

A. Female and male aggregated



B.Female



C. Male

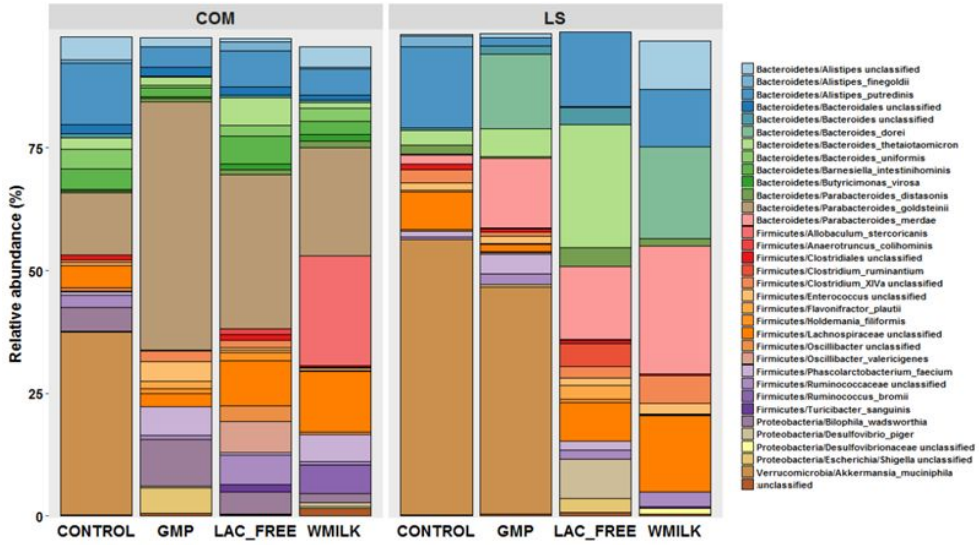


FIGURE S6

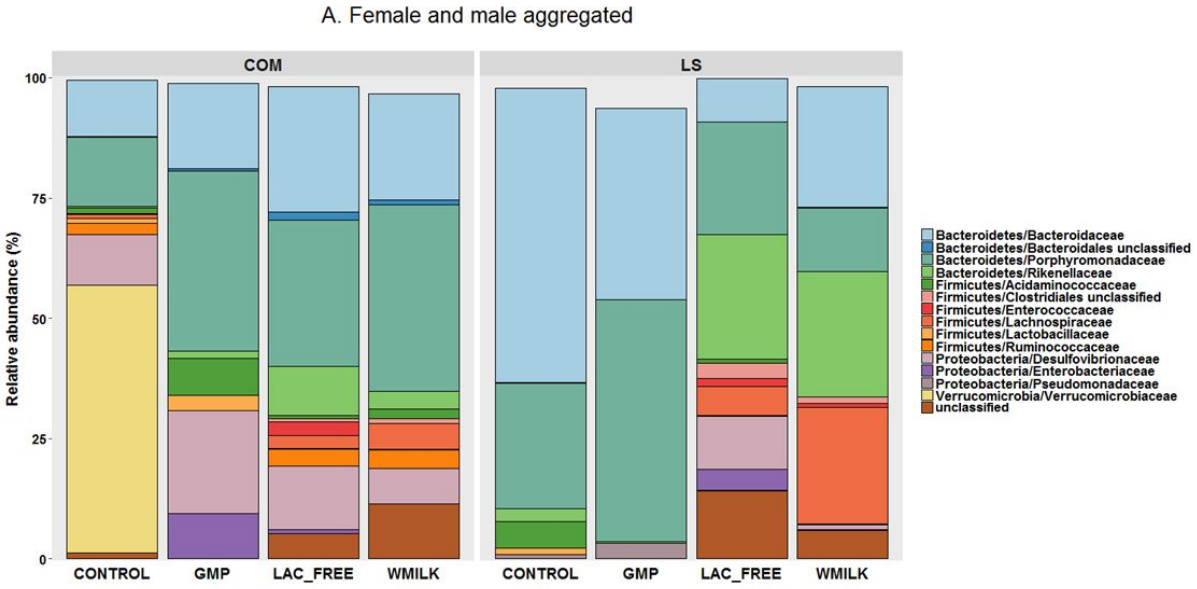
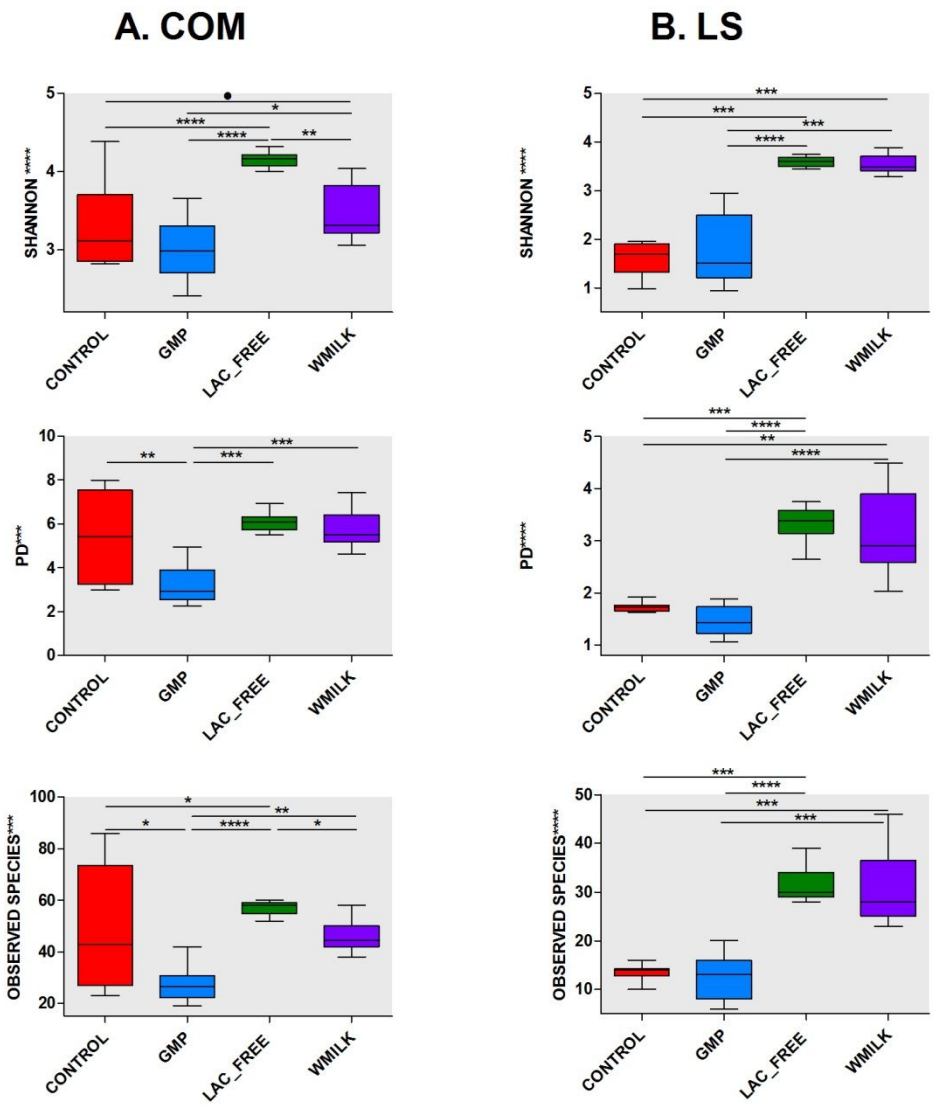

A. Female and male aggregated
COM
LS
Relative abundance (%)
100
75
50
25
0
CONTROL
GMP
LAC_FREE
WMILK
CONTROL
GMP
LAC_FREE
WMILK
Bacteroidetes/Bacteroidaceae
Bacteroidetes/Bacteroidales unclassified
Bacteroidetes/Porphyromonadaceae
Bacteroidetes/Rikenellaceae
Firmicutes/Acidaminococcaceae
Firmicutes/Clostridiales unclassified
Firmicutes/Enterococcaceae
Firmicutes/Lachnospiraceae
Firmicutes/Lactobacillaceae
Firmicutes/Ruminococcaceae
Proteobacteria/Desulfovibrionaceae
Proteobacteria/Enterobacteriaceae
Proteobacteria/Pseudomonadaceae
Verrucomicrobia/Verrucomicrobiaceae
unclassified

FIGURE S7


A. COM
B. LS
SHANNON ****
PD***
OBSERVED SPECIES***
PD****
OBSERVED SPECIES****
CONTROL
GMP
LAC_FREE
WMILK

GRAPHIC FOR TABLE OF CONTENTS